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The Adaptor SAP Controls NK Cell Activation by Regulating the Enzymes Vav-1 and SHIP-1 and by Enhancing Conjugates with Target Cells

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SUMMARY

The adaptor SAP, mutated in X-linked lymphoproliferative disease, has critical roles in multiple immune cell types. Among these, SAP is essential for the ability of natural killer (NK) cells to eliminate abnormal hematopoietic cells. Herein, we elucidated the molecular and cellular bases of this activity. SAP enhanced NK cell responsiveness by a dual molecular mechanism. It coupled SLAM family receptors to the kinase Fyn, which triggered the exchange factor Vav-1 and augmented NK cell activation. SAP also prevented the inhibitory function of SLAM family receptors. This effect was Fyn independent and correlated with uncoupling of SLAM family receptors from the lipid phosphatase SHIP-1. Both mechanisms cooperated to enable conjugate formation with target cells and to stimulate cytotoxicity and cytokine secretion by NK cells. These data showed that SAP secures NK cell activation by a dichotomous molecular mechanism, which is required for conjugate formation. These findings may have implications for the role of SAP in other immune cell types.

INTRODUCTION

Natural killer (NK) cells have a critical function in immune surveillance against transformed and virus-infected cells, in particular hematopoietic cells (Bryceson and Long, 2008; Lanier, 2005; Raulet, 2003; Vivier et al., 2008). Their greater efficiency at reacting against hematopoietic "target" cells is mediated by the signaling lymphocytic activation molecule (SLAM) family, a group of hematopoietic cell-specific receptors named SLAM, 2B4, natural killer, T and B cell antigen (NTB-A; Ly-108 in the mouse), CD2-like receptor activating cytotoxic cells (CRACC), Ly-9, and CD84 (Dong et al., 2009; Engel et al., 2003; Schwartzberg et al., 2009; Veillette, 2006, 2010). With the exception of 2B4, all SLAM family receptors are "self-ligands" (that is, they are their own ligands). 2B4 recognizes as ligand CD48, which is also expressed solely on hematopoietic cells.

SLAM family receptors are not part of a preexisting molecular complex capable of delivering activating signals (Dong et al., 2009; Engel et al., 2003; Schwartzberg et al., 2009; Veillette, 2006, 2010). Rather, they associate in an inducible fashion with a group of Src homology 2 (SH2) domain-only adaptors named the SLAM-associated protein (SAP) family. This family includes SAP, Ewing's sarcoma-activated transcript-2 (EAT-2), and EAT-2-related transducer (ERT). All SAP family adaptors are expressed in NK cells. There is increasing evidence that SAP family adaptors play a critical role in the functions mediated by SLAM family receptors in immune cells, including NK cells.

The importance of SAP in immunity was revealed by the observation that most cases of X-linked lymphoproliferative (XLP) disease, a human immunodeficiency, are due to inactivating mutations in the SAP-encoding gene *SH2D1A* (Engel et al., 2003; Schwartzberg et al., 2009; Veillette, 2006, 2010). It was subsequently shown that SAP regulates multiple immune cell functions in humans and in mice. These include NK cell activation in response to hematopoietic cells, T helper 2 cytokine production, follicular T helper cell functions, $CD8^+$ T cell cytotoxicity, and NK-T cell development. EAT-2 and ERT synergize with SAP to promote NK cell activation in response to hematopoietic cells (Dong et al., 2009).

Because SAP is made up almost only of an SH2 domain, it was initially postulated to promote immune cell functions by preventing coupling of SLAM family receptors to SH2 domain-containing inhibitory molecules such as SH2 domain-containing protein-tyrosine phosphatase-1 (SHP-1), SHP-2, SH2 domain-containing inositol 5′-phosphatase-1 (SHIP-1), and the inhibitory kinase Csk (Dong et al., 2009; Engel et al., 2003; Schwartzberg et al., 2009; Veillette, 2006, 2010). These polypeptides can interact with SLAM family receptors in the absence of SAP, at least in vitro and in transfected cells. Subsequently, it was found that SAP can also couple SLAM family receptors to activating intracellular signals. It is able to recruit the Src family protein tyrosine kinase (PTK) Fyn, by way of a direct interaction between arginine 78 (R78) of SAP and the Src homology 3 (SH3) domain

of Fyn (Chan et al., 2003; Latour et al., 2001, 2003). This interaction appears to be important for some, but not all, of the functions of SAP (Cannons et al., 2004, 2006; Davidson et al., 2004; Nunez-Cruz et al., 2008; Qi et al., 2008). Through R78, SAP can also bind Pak-interacting exchange factor (PIX), the adaptor Nck, and protein kinase C-θ (PKC-θ) (Cannons et al., 2010; Gu et al., 2006; Li et al., 2009). Unlike SAP, EAT-2 and ERT do not possess the R78-based motif. Both seem to mediate alternate signals, involving phosphorylation of tyrosines in their carboxy-terminal tail (Cruz-Munoz et al., 2009; Roncagalli et al., 2005).

The mechanism by which SAP enhances NK cell activation in response to hematopoietic cells is not well understood. It is not established whether SAP promotes NK cell activation through active signaling by Fyn or other SAP-associated effectors, through prevention of inhibitory signaling by SLAM family receptors, or through a combination of these mechanisms. The downstream pathways mediating active signaling by SAP in NK cells are also not clarified. Likewise, the effectors of SLAM family receptor-mediated inhibition, as well as the mechanism by which SAP can prevent this inhibition, are not elucidated. Lastly, whether SAP is promoting NK cell activation by stabilizing contacts with hematopoietic cells or by influencing later steps of NK cell activation is not known.

Herein, we found that SAP promotes NK cell activation by a dual mechanism. On the one hand, SAP couples SLAM family receptors to Fyn, which then links SAP to the exchange factor Vav-1. On the other hand, SAP prevents inhibition by SLAM family receptors. The latter effect is independent of Fyn and appears to relate to uncoupling of SLAM family receptors from SHIP-1, an inhibitor of Ca^{2+} fluxes. Both activities cooperate to enable NK cells to form stable lymphocyte function-associated antigen-1 (LFA-1)-dependent conjugates with target cells and, ultimately, to mediate cytotoxicity and produce cytokines.

RESULTS

Fyn-Binding Site of SAP Is Critical for NK Cell Activation

To determine how SAP controls NK cell activation, we first tested the importance of Fyn recruitment. NK cell functions were examined in a genetically modified mouse in which the Fyn-binding site of SAP, R78, was mutated to alanine (SAP R78A mutation) (Davidson et al., 2004). Like SAP deficiency (Bloch-Queyrat et al., 2005; Dong et al., 2009), the R78A mutation had no effect on NK cell development (Figure 1A), or the expression of SAP, SLAM family receptors, or CD48 (Figure 1B; Figure S1A available online).

Compared to wild-type (WT) NK cells, SAP R78A NK cells had severely decreased killing of the hematopoietic cells RMA-S, YAC-1, and major histocompatibility complex (MHC) class I-deficient splenocytes in vitro (Figure 1C). No defect was observed toward CHO, a hamster-derived nonhematopoietic cell line. These findings were consistent with the notion that SAP is required for the responsiveness of NK cells toward hematopoietic cells, but not toward nonhematopoietic cells (Dong et al., 2009). With RMA-S, the impact of the SAP R78A mutation was similar to that of SAP deficiency, whereas with YAC-1 and splenocytes, it was slightly less severe. We also combined the SAP R78A mutation with EAT-2 deficiency (Figure 1D). Like SAP deficiency, the SAP R78A mutation cooperated with lack of EAT-2 to reduce killing of RMA-S. The influence of the SAP R78A mutation was slightly less pronounced than that of SAP deficiency.

Elimination of hematopoietic cells was also examined by a peritoneal clearance assay (Figure 1E). Compared to WT mice, SAP R78A mice exhibited a ~5-fold increase in residual RMA-S cells in the peritoneal cavity, indicating compromised clearance of RMA-S. SAP-deficient mice had a ~10-fold augmentation of residual RMA-S cells.

The capacity of NK cells to produce interferon-γ (IFN-γ) was also analyzed (Figures 1F and 1G). SAP R78A NK cells exhibited compromised IFN-γ production in response to RMA-S. However, this defect seemed less pronounced than in SAP-deficient NK cells. Less obvious defects were seen toward YAC-1. Unlike RMA-S, YAC-1 expresses ligands for multiple activating receptors other than SLAM family receptors (Bloch-Queyrat et al., 2005; Dong et al., 2009). All NK cells responded well to phorbol myristate acetate (PMA) and ionomycin (Figure S1B).

Therefore, the Fyn-binding site was critical for SAP to promote NK cell cytotoxicity and IFN-γ production in response to hematopoietic cells. However, in the absence of Fyn binding, SAP was still able to cause a small enhancement of NK cell responsiveness.

Fyn-Binding Site Is Necessary for 2B4-Mediated NK Cell Activation

To probe directly the role of the Fyn-binding site of SAP in SLAM family receptor functions, NK cell cytotoxicity was tested against B16 melanoma cells expressing or not ligands for SLAM family receptors (Figures 2A and 2B). As reported (Dong et al., 2009), expression of CD48, the ligand of 2B4, promoted killing of B16 by WT NK cells. This effect was lost in SAP-deficient NK cells. Furthermore, 2B4 was converted into an inhibitory receptor in the absence of SAP. When 2B4 was engaged on SAP-deficient NK cells, cytotoxicity was inhibited by ~20% in comparison to when it was not engaged (Figure 2B). In SAP R78A NK cells, the activating effect of 2B4 was also reduced (Figures 2A and 2B). However, unlike in SAP-deficient NK cells, there was no conversion of 2B4 into an inhibitory receptor. In fact, a residual activating effect (~20%) of 2B4 was present (Figure 2B). These effects were seen both at the high and at the low E:T ratios (Figures 2B and S2).

Because the inhibitory effect of 2B4 in SAP-deficient NK cells was of small magnitude, we postulated that this could be because the ability of SAP to prevent 2B4-triggered inhibition was shared by EAT-2. To address this possibility, 2B4-mediated inhibition was examined in NK cells lacking SAP alone, EAT-2 alone, or both (Figure 2C). The ability of SAP deficiency to convert 2B4 into an inhibitory receptor was much stronger in NK cells also lacking EAT-2. Hence, the capacity of SAP to prevent inhibition by 2B4 was partially masked in the presence of EAT-2.

To understand how Fyn binding might promote 2B4 function, we analyzed tyrosine phosphorylation of Vav-1 and SHIP-1, the two most prominent tyrosine phosphorylation substrates triggered by 2B4 (Figures 2D and 2E; Chen et al., 2004; Mesecke et al., 2011; Munitz et al., 2005). Vav-1 is an exchange factor promoting multiple functions in immune cells, including

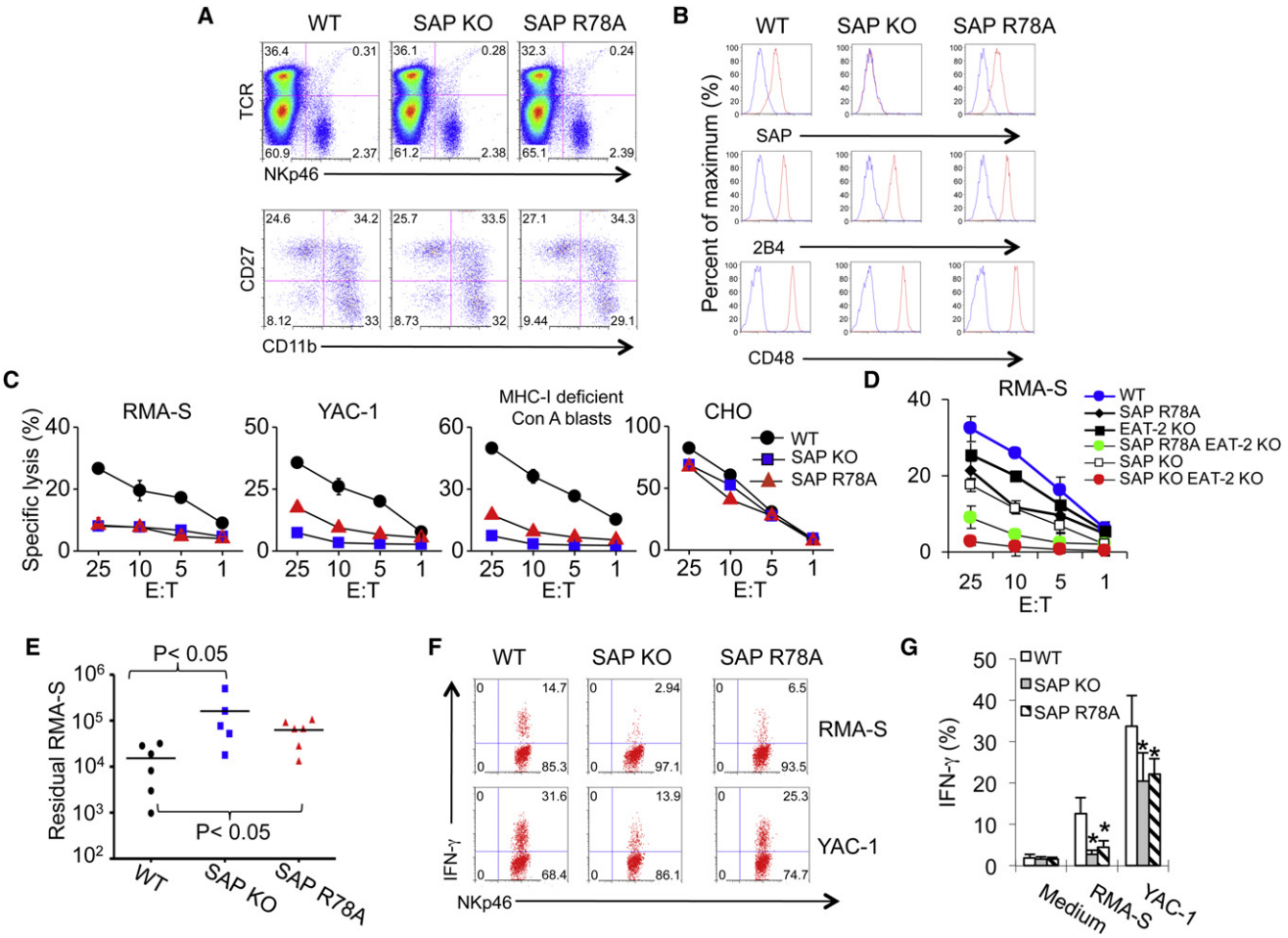

Figure 1. The Fyn-Binding Site of SAP Is Critical for NK Cell Activation

(A) Splenocytes of wild-type (WT), SAP-deficient (KO), and SAP R78A mice were stained with anti-NKp46 and anti-TCRβ (top). NK cells are NKp46$^+$TCRβ$^-$. Proportions of NK cells are shown in the right lower quadrant. Expression of CD11b and CD27 on NKp46$^+$TCRβ$^-$ was also analyzed (bottom). Representative of n = 3.

(B) Expression of SAP, 2B4, and CD48 (red) was analyzed on NKp46$^+$TCRβ$^-$ splenocytes. Isotype controls are shown as the blue line. Representative of n = 3.

(C and D) Natural cytotoxicity was tested with ex vivo splenic NK cells, and the targets RMA-S, YAC-1, class I major histocompatibility (MHC-I)-deficient splenocytes, and CHO. Standard deviations of duplicates are shown by error bars. Representative of at least n = 5 (C) and n = 3 (D).

(E) Green fluorescent protein (GFP)-expressing RMA-S cells were injected intraperitoneally into mice (five to six per group), and the residual RMA-S cells in the peritoneal cavity were counted after 18 hr. Symbols represent individual mice. Mean values are shown as the horizontal lines.

(F and G) Ex vivo NK cells were incubated with RMA-S or YAC-1 for 4 hr. IFN-γ production by NKp46$^+$TCRβ$^-$ was detected by intracellular staining (F). Percentages of IFN-γ-producing are shown in the upper right quadrant. Representative of at least n = 4. Average values with standard deviations from multiple independent experiments are shown in (G). *p < 0.05, compared to WT.

Related to Figure S1.

receptor clustering and cytoskeletal reorganization. In contrast, SHIP-1 is a lipid phosphatase that inhibits immune cell activation, largely through suppression of Ca^{2+} fluxes. Tyrosine phosphorylation of Vav-1 and SHIP-1 correlates with their functional activation.

2B4-evoked tyrosine phosphorylation of Vav-1 was nearly abolished in SAP-deficient and SAP R78A NK cells (Figure 2D). This effect was not seen with SHIP-1. Tyrosine phosphorylation of SHIP-1 was slightly enhanced (~2-fold) in SAP-deficient NK cells. Moreover, as was the case for inhibition of cytotoxicity (Figure 2C), the impact on SHIP-1 was accentuated when SAP deficiency was combined with EAT-2 deficiency (Figure 2E). In comparison, the effect of the SAP R78A mutation on SHIP-1 was disparate (Figure 2D). SHIP-1 phosphorylation was reduced, rather than enhanced, in SAP R78A NK cells. Perhaps SAP R78A, but not SAP deficiency, interfered with coupling of 2B4 to the PTKs mediating SHIP-1 phosphorylation. Note that because SLAM family receptors are self-ligands or, in the case of 2B4, recognizing a ligand expressed on NK cells, alterations in SAP family adaptors can change basal signaling, in addition to signaling in stimulated cells. This is because SLAM family receptors are engaged even in "unstimulated" cells.

The finding that 2B4 was able to couple to SHIP-1 tyrosine phosphorylation in the absence of SAP or other SAP-related adaptors suggested that 2B4 tyrosine phosphorylation, which initiates 2B4 signaling, was not dependent on SAP family

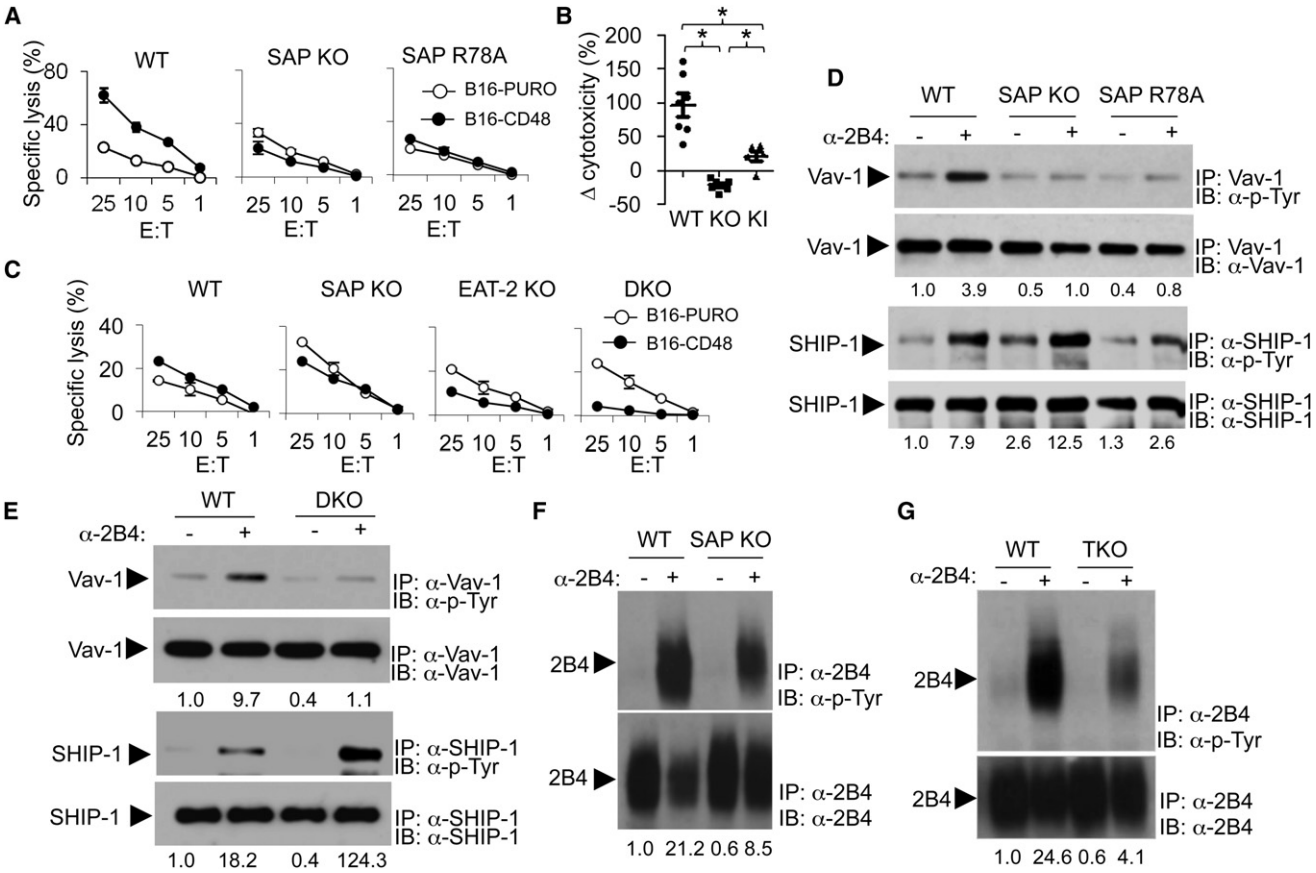

Figure 2. The Fyn-Binding Site of SAP Controls 2B4-Triggered Activation

(A and B) This experiment was performed as described for Figure 1C, except that B16 cells expressing the puromycin resistance marker alone (PURO) or in combination with CD48 were used (A). Standard deviations of duplicates are shown by error bars. Representative of at least n = 5. The difference in cytotoxicity (Δcytotoxicity) toward B16 expressing CD48 versus B16 lacking CD48 was calculated at the 25:1 E:T ratio (B). Statistics for the other E:T ratios are shown in Figure S2. Symbols represent individual mice. KO, SAP-deficient mice; KI, SAP R78A mice. *$p < 0.01$.

(C) Ex vivo NK cells were tested in natural cytotoxicity assays as detailed for (A). DKO, double SAP-EAT-2 knockout mice. Representative of n = 3.

(D) IL-2-expanded NK cells were stimulated for 2 min with anti-2B4. Tyrosine phosphorylation of Vav-1 and SHIP-1 was analyzed. Quantitation of relative tyrosine phosphorylation is shown at the bottom. Representative of at least n = 5.

(E) This experiment was performed as detailed for (D). Representative of n = 3.

(F and G) The experiments were performed as for (D), except that 2B4 was immunoprecipitated. Representative of at least n = 5 (F) and n = 3 (G). Related to Figure S2.

adaptors. This possibility was intriguing, given that, in T cells, tyrosine phosphorylation of SLAM family receptors, including 2B4, is firmly dependent on SAP (Chen et al., 2004; Latour et al., 2001; Simarro et al., 2004; Zhong and Veillette, 2008). To address this idea, 2B4 tyrosine phosphorylation was analyzed in SAP-deficient NK cells (Figure 2F). Although 2B4 tyrosine phosphorylation was reduced, some tyrosine phosphorylation of 2B4 was observed. To ascertain whether this residual phosphorylation was due to the other SAP-related adaptors, similar experiments were conducted with NK cells lacking all three SAP-related adaptors (Figure 2G). Although diminished, tyrosine phosphorylation of 2B4 was still seen in NK cells lacking all SAP family adaptors.

Thus, through its Fyn-binding site, SAP was required for 2B4 to trigger Vav-1 and stimulate NK cell activation. EAT-2 could not replace SAP in this function, presumably because EAT-2 does not have the R78-based motif. SAP was also able to suppress SHIP-1 and prevent the inhibitory effect of 2B4 on NK cell activation. The latter effect was independent of Fyn binding and was shared by EAT-2. Lastly, SAP and the other SAP-related adaptors were not strictly required for 2B4 tyrosine phosphorylation in NK cells.

Fyn Is Required for Activating Function of SLAM Family Receptors

Because R78 of SAP can also mediate associations with PIX, Nck, and PKC-θ (Cannons et al., 2010; Gu et al., 2006; Li et al., 2009), we ascertained whether Fyn was the critical effector of R78 by performing analogous studies with Fyn-deficient NK cells (Figure 3A). Fyn-deficient NK cells, as well as Lck-deficient NK cells used as controls, had normal levels of SAP, SLAM family receptors, and CD48 (Figures 3B and S3A). However, Fyn-deficient NK cells exhibited defective killing of RMA-S, B16, and, to a lesser extent, YAC-1 (Figures 3C and 3F;

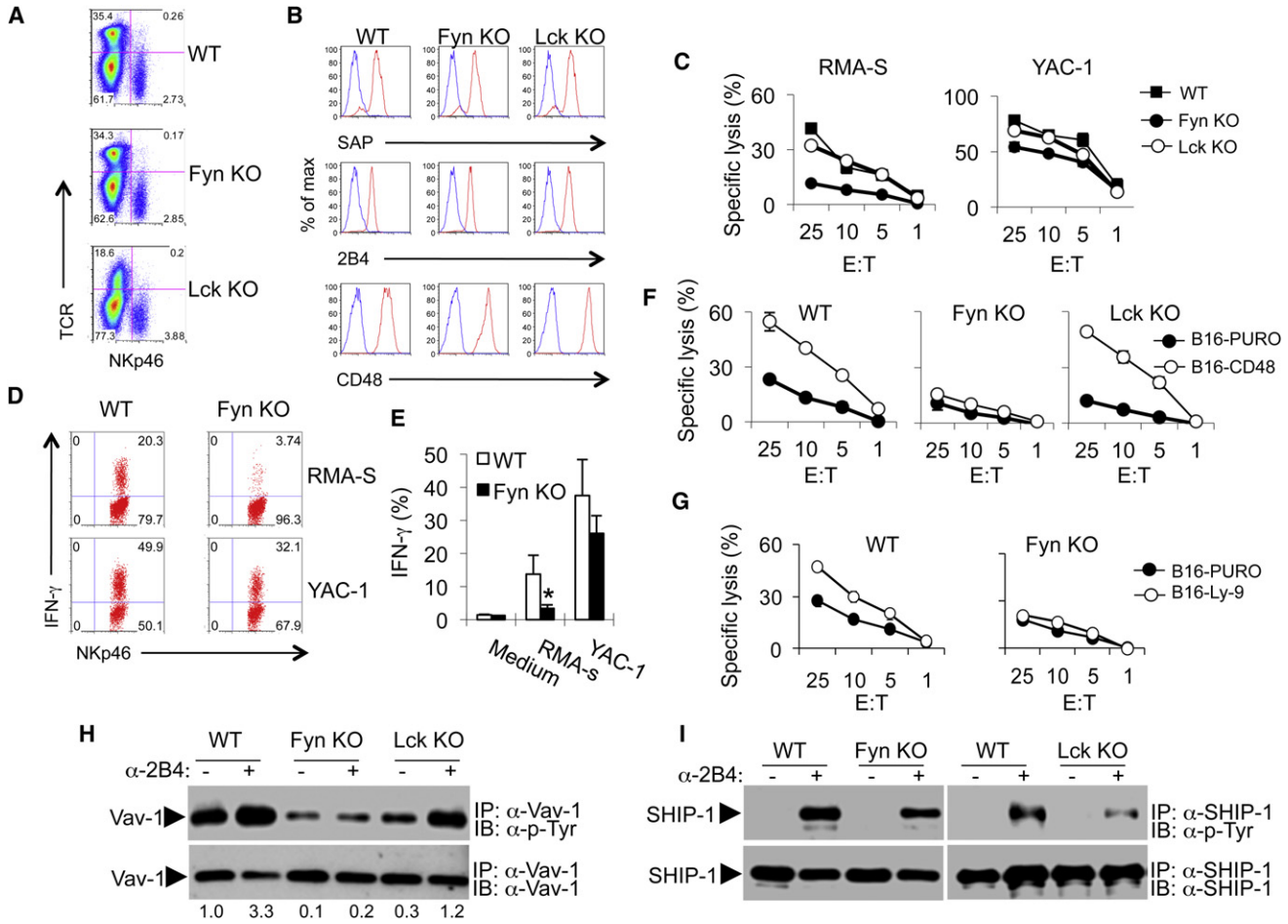

Figure 3. Fyn Is Required for the Activating Function of SLAM Family Receptors

(A) Splenocytes were stained with anti-NKp46 and anti-TCRβ. NK cells are NKp46$^+$TCRβ$^-$. Proportions of NK cells are shown in the right lower quadrant. Note that the proportion of T cells (NKp46$^+$TCRβ$^-$) is reduced in Lck-deficient (KO) mice, in keeping with the block in thymocyte development in these animals (Molina et al., 1992). Representative of n = 3.

(B) Expression of SAP, 2B4, and CD48 (red) was analyzed for NKp46$^+$TCRβ$^-$ splenocytes. Isotype controls are shown as the blue line. Representative of n = 3.

(C, F, and G) Ex vivo NK cells were tested in natural cytotoxicity assays, using as targets RMA-S and YAC-1 (C), B16 expressing or not CD48 (F), or B16 expressing or not Ly-9 (G). Standard deviations of duplicate values are shown as error bars. Representative of n = 5 (C), n = 3 (F), and n = 4 (G).

(D and E) Ex vivo NK cells were incubated with RMA-S or YAC-1 for 4 hr. IFN-γ production by NKp46$^+$TCRβ$^-$ was detected by intracellular staining (D). Percentages of IFN-γ-producing are shown in the upper right quadrant. Representative of n = 3. Average values with standard deviations from multiple independent experiments are shown in (E). *$p < 0.05$.

(H and I) IL-2-expanded NK cells were stimulated for 2 min with anti-2B4. Tyrosine phosphorylation of Vav-1 (H) and SHIP-1 (I) was analyzed. Quantitation of relative tyrosine phosphorylation is shown at the bottom. Quantitation was not possible for (I), because no tyrosine phosphorylated band was detected in unstimulated lanes. Representative of n = 4.

Related to Figure S3.

Lowin-Kropf et al., 2002). They also had reduced IFN-γ production in response to RMA-S (Figures 3D, 3E, and S3B). Thus, Fyn was required for NK cell activation by hematopoietic and nonhematopoietic cells.

More importantly, Fyn, but not Lck, was needed for the activating effect of 2B4 toward cytotoxicity (Figure 3F). However, unlike SAP deficiency, lack of Fyn did not result in conversion of 2B4 into an inhibitory receptor (Figures 3F and 3G). Fyn was also required for the activating function of Ly-9, another SLAM family member (Figure 3G). Likewise, it was needed for 2B4-triggered Vav-1 tyrosine phosphorylation (Figure 3H). Although a partial reduction of Vav-1 tyrosine phosphorylation was seen in Lck-deficient NK cells, this decrease was equally observed in the absence of 2B4 stimulation. Consequently, Lck did not affect the extent of induction of Vav-1 phosphorylation upon 2B4 triggering. In the case of SHIP-1, tyrosine phosphorylation was partially compromised in both Fyn-deficient and Lck-deficient NK cells (Figure 3I).

Hence, Fyn was necessary for SLAM family receptors to trigger Vav-1 and augment NK cell activation. However, Fyn was not needed for SAP to prevent SLAM family receptor-mediated inhibition. Nonetheless, Fyn (presumably the pool not associated with SAP), in combination with Lck, was needed for tyrosine phosphorylation of SHIP-1 by 2B4 engagement.

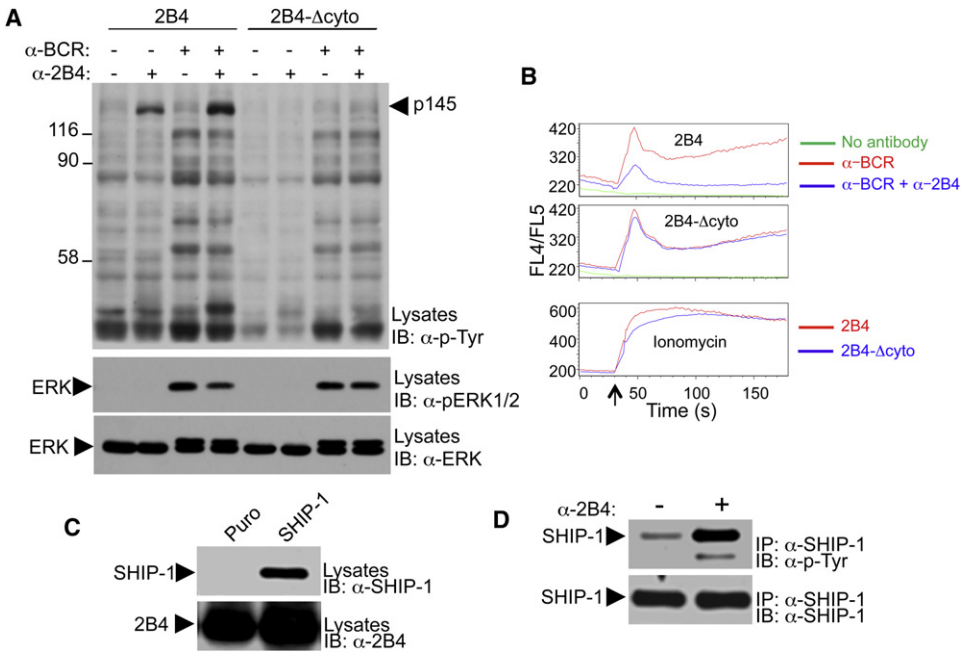

Figure 4. Reconstitution of 2B4-Mediated Inhibition in DT-40 Cells

(A) DT-40 cells expressing full-length mouse 2B4 or cytoplasmic domain-truncated mouse 2B4 (2B4-Δcyto) were stimulated with B cell receptor (BCR) or 2B4 antibodies, alone or in combination. Protein tyrosine phosphorylation and Erk activation were analyzed. The migrations of molecular weight markers are shown on the left and that of p145 is indicated on the right. Representative of n = 3.

(B) Cells expressing 2B4 or 2B4-Δcyto were loaded with Indo-1 and stimulated with anti-BCR, without or with anti-2B4, or ionomycin. Changes in intracellular Ca^{2+} were monitored by flow cytometry, with the FL4/FL5 fluorescence ratio. Representative of n = 5.

(C) SHIP-1-deficient DT-40 cells, expressing mouse 2B4, were transfected with a plasmid encoding the puromycin marker alone (Puro) or in combination with mouse SHIP-1. Cell lysates were immunoblotted with SHIP-1 or 2B4 antibodies. Representative of n = 3.

(D) SHIP-1-reconstituted DT-40 cells, expressing mouse 2B4, were stimulated or not with anti-2B4. Tyrosine phosphorylation of SHIP-1 was evaluated. Representative of n = 3.

Related to Figure S4.

Reconstitution of 2B4-Mediated Inhibition in DT-40 Cells

We ascertained whether SHIP-1 or, alternatively, other inhibitory polypeptides like SHP-1, SHP-2, and Csk was (were) responsible for 2B4-mediated inhibition (Dong et al., 2009; Parolini et al., 2000; Sivori et al., 2000). One difficulty in addressing this issue is that NK cell development is severely altered in SHIP-1-deficient mice (Wahle et al., 2007; Wang et al., 2002). To circumvent this problem, we sought a system in which 2B4-mediated inhibition could be examined in the absence of developmental defects. DT-40 is a B cell line lacking SAP and Fyn (Hata et al., 1994; Kurosaki, 1999; Mikhalap et al., 2004; Pearse et al., 1999; Takata et al., 1994) and for which variants lacking SHIP-1, SHP-1, SHP-2, SHP-1 plus SHP-2, or Csk are available (Hata et al., 1994; Kurosaki, 1999; Mikhalap et al., 2004; Pearse et al., 1999; Takata et al., 1994).

To ascertain whether 2B4 was inhibitory in DT-40, derivatives expressing full-length 2B4 or a 2B4 variant lacking the cytoplasmic domain (2B4-Δcyto) were generated (Figures S4A and S4B; data not shown). Stimulation of full-length 2B4 induced tyrosine phosphorylation of a 145 kDa polypeptide (p145) consistent with SHIP-1 (Figure 4A). Moreover, it induced tyrosine phosphorylation of 2B4 (Figure S4C). When 2B4 was coaggregated with the B cell receptor (BCR), an immunoreceptor tyrosine-based activation motif (ITAM)-coupled receptor, there was little or no decrease in BCR-triggered protein tyrosine phosphorylation. However, there was inhibition of BCR-triggered Ca^{2+} fluxes and, to a weaker extent, Erk activation (Figures 4A and 4B). These effects were similar to those previously seen in NK cells lacking SAP family adaptors, upon coaggregation of 2B4 with the ITAM-coupled receptor CD16 (Dong et al., 2009). No signals were caused by 2B4-Δcyto (Figures 4A and 4B). To assess whether p145 was SHIP-1, mouse SHIP-1 was expressed in a SHIP-1-deficient variant of DT-40 cells (Figure 4C). Immunoprecipitation with antibodies against mouse SHIP-1 indicated that p145 was SHIP-1 (Figure 4D). Therefore, engagement of 2B4 on DT-40 resulted in biochemical effects similar to the ones seen in NK cells lacking SAP family adaptors.

Central Role of SHIP-1 in 2B4-Mediated Inhibition

Analogous experiments were performed with DT-40 lacking SHIP-1, SHP-1, SHP-2, or Csk (Figures 5, S4A, and S4B). Because the most dramatic inhibitory effect of 2B4 in DT-40 was on Ca^{2+} fluxes, these studies concentrated on Ca^{2+} responses (Figures 5A and 5B). Lack of SHP-1, SHP-2, or Csk had little impact on the inhibitory effect of 2B4. A small reduction of the inhibitory effect of 2B4 (to ~75% of control) was seen in cells lacking both SHP-1 and SHP-2. However, in cells lacking SHIP-1, there was a prominent diminution of the inhibitory

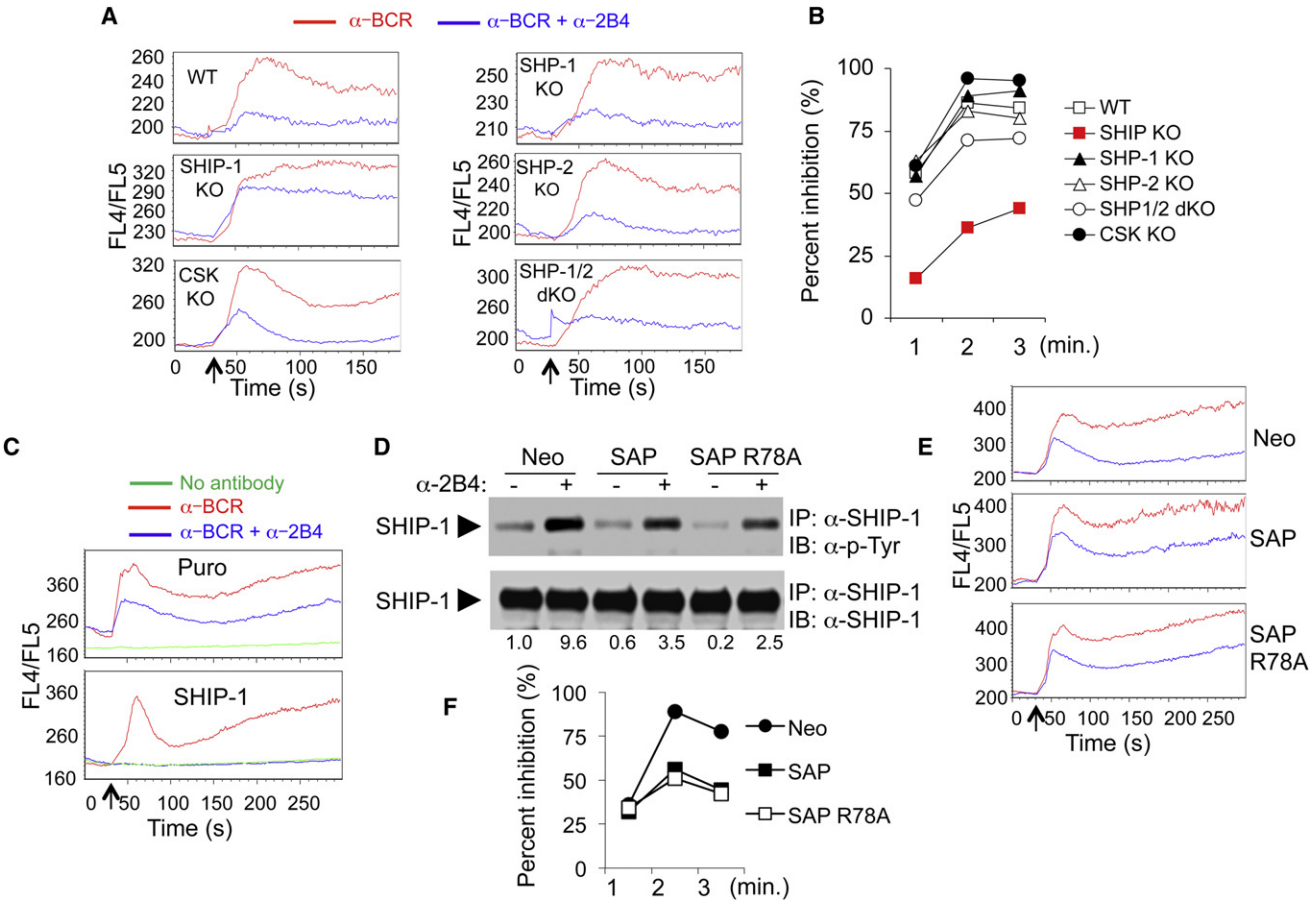

Figure 5. Role of SHIP-1 in 2B4-Mediated Inhibition in DT-40 Cells

(A and B) 2B4-expressing DT-40 cells deficient in SHIP-1, Csk, SHP-1, SHP-2, or SHP-1 and SHP-2 were stimulated with anti-BCR with or without anti-2B4. Ca^{2+} fluxes were examined as detailed for Figure 4B (A). A quantitation of the percentage of inhibition of Ca^{2+} levels in cells stimulated with anti-2B4 plus anti-BCR, in comparison to anti-BCR alone, at different times is represented graphically (B). Representative of n = 4.

(C) SHIP-1-deficient DT-40 cells, reconstituted or not with mouse SHIP-1, were assayed for 2B4-mediated inhibition as detailed for Figure 4B. Representative of n = 4.

(D–F) 2B4-positive DT-40 cells expressing mouse SHIP-1 were infected with retroviruses encoding the neomycin resistance marker alone (Neo) or in combination with wild-type SAP or SAP R78A. 2B4-evoked tyrosine phosphorylation of SHIP-1 was examined as for Figure 4D (D). Quantitation of relative tyrosine phosphorylation is shown at the bottom. 2B4-mediated inhibition of Ca^{2+} fluxes was studied as for (A) and (B) (E and F). Representative of n = 4 (D) and n = 3 (E and F). Related to Figure S4.

influence of 2B4 (to ~25% of control). 2B4-mediated inhibition was restored when SHIP-1 was reintroduced in SHIP-1-deficient cells (Figure 5C).

We also utilized DT-40 to ascertain the impact of SAP on 2B4-mediated inhibition (Figures 5D–5F). SAP, either WT or R78A, was introduced in 2B4-expressing DT-40 (Figure 5E). Because Fyn is not expressed in DT-40 (Takata et al., 1994), the impact of the two SAP polypeptides was expected to be similar. Either SAP polypeptide decreased the extent of SHIP-1 tyrosine phosphorylation in response to 2B4 stimulation (Figure 5D). This was accompanied by a reduction of the inhibitory effect of 2B4 on BCR-stimulated Ca^{2+} fluxes (Figures 5E and 5F).

Thus, in DT-40, 2B4-mediated inhibition was mediated by SHIP-1 and, perhaps, SHP-1 and SHP-2. SAP prevented to some degree the inhibitory effect of 2B4, through a Fyn-independent mechanism, and this influence correlated with a decrease in 2B4-evoked SHIP-1 tyrosine phosphorylation.

SAP Enables Conjugate Formation with Target Cells

SAP promotes T cell-dependent B cell responses at least in part by stabilizing conjugate formation between T cells and B cells (Qi et al., 2008). Formation of conjugates between NK cells and target cells is also critical for NK cell activation (Bryceson and Long, 2008; Lanier, 2005; Raulet, 2003; Vivier et al., 2008). We therefore ascertained whether SAP was required for the capacity of NK cells to form conjugates with target cells. Compared to WT NK cells, those lacking SAP displayed markedly reduced conjugate formation with RMA-S (Figure 6A). To assess the role of SAP-associated Fyn in conjugate formation, similar experiments were conducted with NK cells from SAP R78A or Fyn-deficient mice (Figures 6A and 6B). Compared to SAP-deficient NK cells, SAP R78A and Fyn-deficient NK cells exhibited a less pronounced diminution of conjugate formation.

Interactions between the adhesion molecules LFA-1 and intercellular adhesion molecule-1 (ICAM-1) are critical for

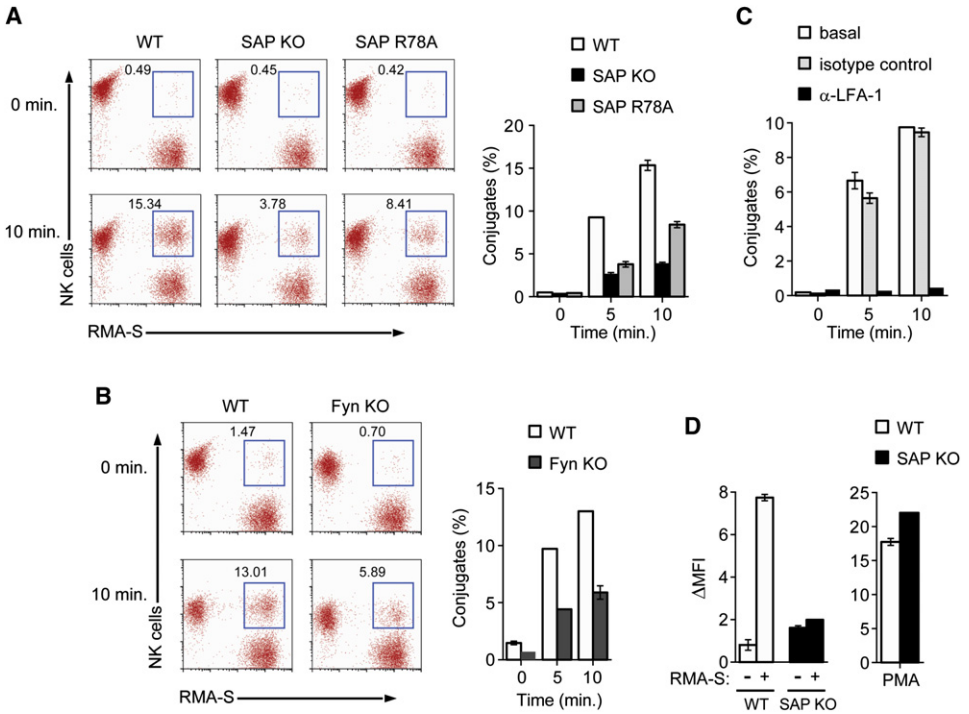

Figure 6. Requirement of SAP Pathway for Conjugate Formation and LFA-1 Activation

(A and B) IL-2-expanded NK cells labeled with PE-Cy7-conjugated anti-NK1.1 were incubated with GFP-expressing RMA-S. Conjugate formation was examined after 0, 5, and 10 min. Left: Representative experiments and time points are shown. NK cell-target cell conjugates are identified as $NK1.1^{+}GFP^{+}$ cells and are boxed. The percentages of conjugates formed are shown at the top. Right: A graphic representation, with standard deviations of duplicate values, of conjugate formation at different times is shown. Representative of n = 5 (A) and n = 4 (B).

(C) The ability of wild-type NK cells to form conjugates with RMA-S was tested in the presence of blocking LFA-1 antibodies or an irrelevant isotype control. A graphic representation, with standard deviations of duplicate values, of conjugate formation at different times is shown. Representative of n = 3.

(D) The ability of NK cells to bind a soluble ICAM-1-Fc fusion or a human IgG_1 (isotype) control was tested as outlined in Experimental Procedures. ΔMFI is the difference in mean fluorescent intensity (MFI) between cells stained with ICAM-Fc and isotype control. The resulting values are depicted. MFIs and representative histograms are shown in Figure S5C. As control, cells were stimulated with phorbol myristate acetate (PMA; 2 μM). Standard deviations of duplicate values are shown. Representative of n = 3.

Related to Figure S5.

conjugate formation between NK cells and target cells (Bryceson et al., 2009; Hoffmann et al., 2011; Osman et al., 2007). In keeping with this, the ability of normal mouse NK cells to form conjugates with RMA-S was prevented by blocking LFA-1 antibodies (Figures 6C and S5A). Because engagement of 2B4 on human NK cells was previously shown to enhance LFA-1-dependent adhesion by augmenting the affinity of LFA-1 for ICAM-1 (Hoffmann et al., 2011), we tested whether SAP promoted an increase in the affinity of LFA-1 (Figures 6D, S5B, and S5C). Unlike the situation in humans, there is no available antibody that recognizes "activated" LFA-1 molecules in the mouse. Thus, these experiments were done with soluble ICAM-1 as a probe (Kinashi, 2005). Exposure of WT NK cells to RMA-S resulted in an increase in binding to soluble ICAM-1. This effect was nearly abolished in SAP-deficient NK cells. Although the magnitude of the increase in ICAM-1 binding in these assays may seem small, this is typical for such experiments, because only a small fraction of LFA-1 is activated upon cell stimulation (Baker et al., 2009; Davidson et al., 2010; Stewart et al., 1996). WT and SAP-deficient NK cells expressed similar levels of LFA-1, and they underwent similar increases in ICAM-1 binding in response to PMA (Figures 6D, S5B, and S5C).

Hence, SAP was necessary for conjugate formation between NK cells and RMA-S. This function was partially Fyn dependent and partially Fyn independent. Moreover, SAP was required for NK cells to undergo an increase in LFA-1 affinity in response to RMA-S.

Roles of Vav, Phospholipase C-γ2, and Calcium in Conjugate Formation

The data above implied that SAP acted both by activating Vav-1 (through Fyn) and by preventing inhibition via SHIP-1 (independently of Fyn). Hence, we ascertained whether Vav and the SHIP-1 targets, phospholipase (PLC)-γ2 and Ca^{2+} fluxes, were needed for conjugate formation (Figure 7). To address the role of Vav, we analyzed conjugate formation by NK cells lacking all three Vav proteins (hereafter termed Vav-deficient NK cells) (Figure 7A). Vav-deficient NK cells exhibited nearly abolished conjugate formation with RMA-S. In agreement with a previous report (Cella et al., 2004), they also had nearly abolished cytotoxicity toward RMA-S and YAC-1 (Figure S6A). Vav proteins are

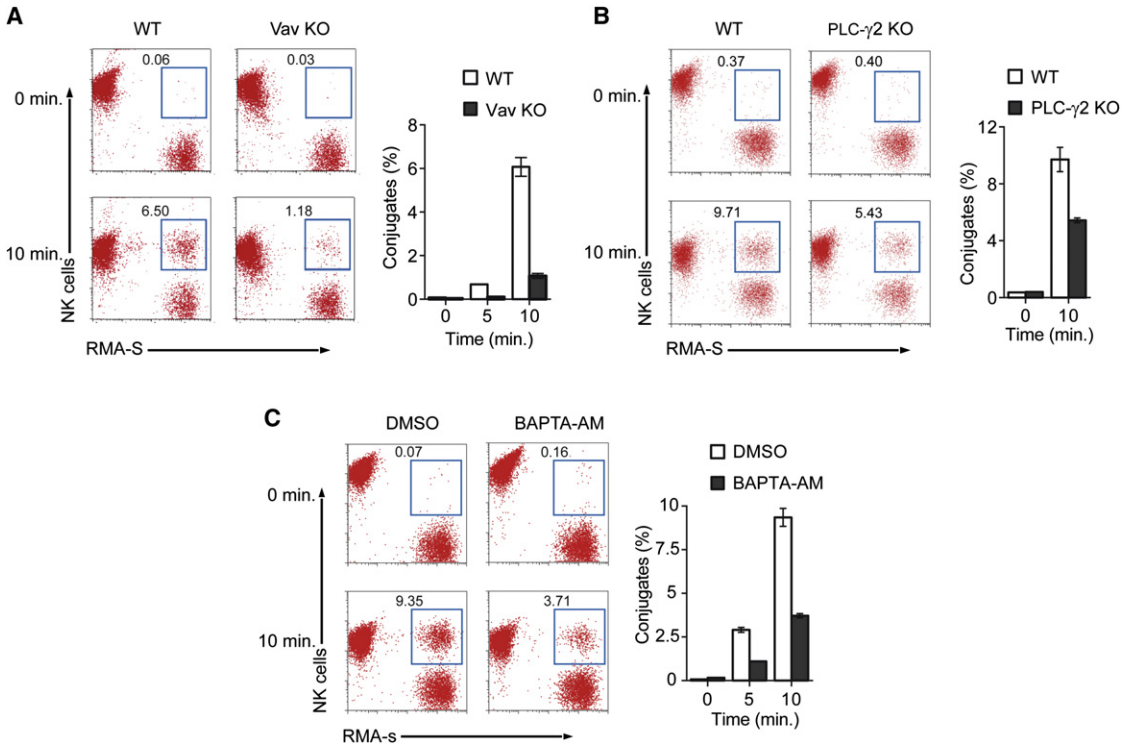

Figure 7. Vav, Phospholipase C-γ2, and Calcium Are Critical for Conjugate Formation

Conjugate formation was analyzed as detailed for Figure 6 (left). A graphic representation, with standard deviations of duplicate values, of conjugate formation at different times is shown on the right.

(A) Vav-deficient cells. Representative of n = 3.

(B) PLC-γ2-deficient NK cells. Representative of n = 3.

(C) BAPTA-AM treatment. NK cells from wild-type mice were incubated with BAPTA-AM (in DMSO) or DMSO alone for 30 min. They were subsequently washed and processed for conjugate formation. Representative of n = 3.

Related to Figure S6.

involved in the activating function of multiple receptors including SLAM family receptors.

To test the role of PLC-γ2, NK cells were obtained from an inducible PLC-γ2-deficient mouse (Figure S6B; Hashimoto et al., 2000). PLC-γ2-deficient cells displayed a partial reduction of conjugate formation with RMA-S (Figure 7B). Likewise, they had reduced ability to kill RMA-S and YAC-1 (Figure S6D). Like NK cells from constitutive PLC-γ2-deficient mice (Caraux et al., 2006b; Regunathan et al., 2006; Tassi et al., 2005a), they exhibited normal expression of most cell surface markers with the exception of CD11b and Ly49G2 (Figure S6C). Thus, to ensure that the effects of PLC-γ2 deficiency were not due to altered development, we also examined the influence of 1,2-bis(2-aminophenoxy)ethane N,N,N',N'-tetraacetic acid acetoxymethyl ester (BAPTA-AM), a chelator of intracellular Ca^{2+} (Figure 7C). BAPTA-AM provoked a partial reduction of conjugate formation with RMA-S by WT NK cells (Figure 7C). It completely abrogated CD16- or ionomycin-induced Ca^{2+} fluxes (Figure S6E). Moreover, it reduced killing of RMA-S, although no effect was seen on YAC-1 (Figure S6F).

Therefore, Vav played a crucial role in promoting conjugate formation with target cells. PLC-γ2 and Ca^{2+} fluxes had a lesser impact in this process.

DISCUSSION

The molecular and cellular mechanism(s) by which SAP promotes NK cell activation are not well understood. To address these issues, we first studied NK cell functions in mice expressing SAP R78A, which cannot be coupled to Fyn, and in mice lacking Fyn. The SAP-Fyn pathway was necessary for NK cell-mediated cytotoxicity toward hematopoietic target cells. It was also essential for enhanced killing of nonhematopoietic targets upon ectopic expression of SLAM family receptor ligands on these targets. Lastly, it was needed for cytokine production in response to hematopoietic target cells.

To identify the immediate effectors of SAP-Fyn, we ascertained tyrosine phosphorylation of Vav-1 (Billadeau et al., 1998; Chan et al., 2001; Colucci et al., 2001; Hornstein et al., 2004; Mesecke et al., 2011). The ability of 2B4 to stimulate Vav-1 tyrosine phosphorylation was nearly abrogated in SAP-deficient NK cells, SAP R78A NK cells, and Fyn-deficient NK cells, but not in Lck-deficient NK cells. Hence, the SAP-Fyn pathway was coupling 2B4 to Vav-1. The other SAP family adaptors expressed in NK cells, EAT-2 and ERT, were unable to substitute for SAP in this function, presumably because they do not have the R78-based motif.

Contrary to the effect of SAP on the activating function of SLAM family receptors, the capacity of SAP to prevent the inhibitory influence of these receptors was not dependent on Fyn. This was documented by assessing 2B4-mediated inhibition in SAP-deficient, SAP R78A, and Fyn-deficient NK cells with non-hematopoietic target cells expressing or not the ligand of 2B4. The prevention of inhibition was paralleled by an interference with tyrosine phosphorylation of SHIP-1. Although the effects of SAP deficiency on SLAM family receptor-mediated inhibition and SHIP-1 tyrosine phosphorylation were small, these effects were much more robust when SAP deficiency was combined with EAT-2 deficiency.

The finding that 2B4 induced SHIP-1 tyrosine phosphorylation in the absence of SAP and other SAP-related adaptors raised the possibility that SAP family adaptors were not needed for 2B4 tyrosine phosphorylation, which initiates 2B4 signaling. In agreement with this, the ability of 2B4 to undergo tyrosine phosphorylation was partially preserved in NK cells lacking all SAP family adaptors. This finding was in striking contrast to those made in T cells, in which SAP was absolutely needed for tyrosine phosphorylation of SLAM family receptors (Chen et al., 2004; Latour et al., 2001; Simarro et al., 2004; Zhong and Veillette, 2008). Furthermore, in T cells, SAP was necessary for tyrosine phosphorylation of SHIP-1 upon engagement of SLAM family receptors. This difference presumably relates to inherently more efficient coupling of SLAM family receptors to PTKs, probably Src family kinases, in NK cells.

To address whether SHIP-1 was responsible for inhibition by SLAM family receptors, we took advantage of the DT-40 cell line in which we were able to recreate the inhibitory effects of 2B4 (Dong et al., 2009). Engagement of 2B4 on DT-40 evoked tyrosine phosphorylation of SHIP-1, and such an effect was attenuated by SAP. Furthermore, investigations with SHIP-1-deficient DT-40 indicated that SHIP-1 was primarily responsible for 2B4-mediated inhibition. SHIP-1 inhibits Ca^{2+} fluxes, the most noticeable target of 2B4-mediated inhibition, as a result of its ability to inactivate Btk family kinases and PLC-γ (Bolland et al., 1998). Because all aspects of 2B4-mediated inhibition seen in NK cells were recreated in DT-40, it seems likely that SHIP-1 was also responsible for 2B4-mediated inhibition in NK cells.

The finding that 2B4 engagement resulted in some SHIP-1 tyrosine phosphorylation in normal NK cells (albeit more weakly than in NK cells lacking SAP family adaptors) suggested that SLAM family receptors elicit inhibitory signals even in normal cells. This inhibitory activity would presumably restrict the activating impact of SLAM family receptors, although the net effect of SLAM family receptor engagement would still be activating. However, one may imagine situations in which the expression or activity of SHIP-1, or those of activating effectors such as SAP, Fyn, or Vav-1, could be altered in normal cells, thereby modifying the net impact of SLAM family receptor engagement. In this manner, SLAM family receptors could act as rheostats of NK cell activation.

Although SHIP-1 seemed to be the primary effector of 2B4-mediated inhibition, ~25% of the inhibitory impact of 2B4 remained in SHIP-1-deficient DT-40 cells. This residual inhibitory activity might be mediated by SHIP-2, another SHIP family member. Alternatively, it might be caused by SHP-1 and SHP-2. In keeping with the latter possibility, combined lack of SHP-1 and SHP-2 in DT-40 resulted in a partial loss (of ~25%) of the inhibitory influence of 2B4.

SAP deficiency caused a near total reduction of the ability of NK cells to form conjugates with target cells. Because conjugate formation is needed for efficient NK cell activation and delivery of perforin-granzyme granules to target cells (Bryceson and Long, 2008; Lanier, 2005; Raulet, 2003; Vivier et al., 2008), these results implied that SAP promotes NK cell activation at least in part by regulating this process. A similar effect of SAP on conjugate formation was previously shown during T cell-B cell interactions (Qi et al., 2008).

Further analyses of SAP R78A and Fyn-deficient NK cells showed that conjugate formation was partially reliant on the SAP-Fyn interaction. This Fyn-dependent activity probably related to activation of Vav, given that Vav-deficient NK cells also had a pronounced defect in conjugate formation. However, part of the function of SAP was also Fyn independent and was probably related to the capacity of SAP to prevent coupling of SLAM family receptors to SHIP-1. Indeed, removal of PLC-γ2 or intracellular Ca^{2+}, two targets of SHIP-1-mediated inhibition, resulted in a partial loss of conjugate formation.

The ability of NK cells to form conjugates with target cells is strongly dependent on interactions between LFA-1 on NK cells and ICAM-1 on target cells (Bryceson et al., 2009; Hoffmann et al., 2011; Osman et al., 2007). Therefore, we tested whether SAP was required to upregulate the affinity of LFA-1 for ICAM-1 during NK cell activation. Experiments with soluble ICAM-1 as a probe indicated that SAP was required for the increased affinity of LFA-1 during NK cell activation. Thus, part of the impact of SAP on conjugate formation was probably mediated via the ability of SAP to enhance the affinity of LFA-1. This might be caused by Fyn-mediated activation of Vav, a known regulator of LFA-1 function.

Although we believe that SAP enhances NK cell activation in significant part by stimulating conjugate formation, it is possible that SAP also promotes other steps of NK cell activation, such as cytoskeletal reorganization, granule exocytosis, and transcription of cytokine genes. Vav, PLC-γ2, and Ca^{2+} fluxes were all implicated in these processes (Caraux et al., 2006a; Tassi et al., 2005b; Hornstein et al., 2004; Swat and Fujikawa, 2005; Turner and Billadeau, 2002).

The findings reported herein provide several new insights into the molecular and cellular mechanisms by which SAP controls NK cell activation. They show that SAP promotes NK cell activation via a dichotomous mechanism (Figure S6G). First, SAP couples SLAM family receptors to Fyn, which, in turn, phosphorylates Vav. Second, SAP interferes with the inhibitory function of SLAM family receptors. This effect is Fyn independent and seemingly relates to the ability of SAP to prevent recruitment of SHIP-1, a negative regulator of PLC-γ-mediated Ca^{2+} fluxes. Coupling of SAP to Vav, together with prevention of the inhibitory function of SLAM family receptors, promotes conjugate formation with target cells and enables greater NK cell-mediated cytotoxicity and cytokine production in response to hematopoietic target cells. Given that the effectors of SAP observed in NK cells are also expressed in other immune cells, it seems likely that a related mechanism explains the ability of SAP to promote other immune cell functions.

EXPERIMENTAL PROCEDURES

NK Cell Assays

Poly(I:C)-activated NK cells or IL-2-expanded NK cells were generated as outlined (Dong et al., 2009). NK cell-mediated cytotoxicity, IFN-γ production, and RMA-S tumor rejection were assayed as detailed (Dong et al., 2009). Poly(I:C)-activated NK cells were used for all in vitro experiments, with the exception of those involving 2B4 signaling or conjugate formation (and the cytotoxicity and Ca^{2+} assays performed as controls for conjugate formation), where IL-2-expanded NK cells were used.

Conjugate Formation

IL-2-activated NK cells (10^6 in 100 μl of serum-free RPMI 1640 medium) were incubated for 30 min on ice with PE-Cy7-conjugated NK1.1 mAb. They were then washed and resuspended at 2×10^6 cells per ml. 100 μl of cell suspension was then added to 100 μl of GFP-positive RMA-S cells (at 2×10^6 cells per ml) and centrifuged at 1,500 rpm (4°C). After removing 150 μl of supernatant, cells were stimulated by incubation at 37°C for 0, 5, or 10 min. Reactions were stopped by adding ice-cold phosphate-buffered saline. Conjugates were detected by flow cytometry. They were $NK1.1^+GFP^+$. To chelate intracellular Ca^{2+}, NK cells were incubated for 30 min with BAPTA-AM (25 mM stock in dimethylsulfoxide [DMSO]; final concentration 40 μM). They were then washed and subjected to conjugate formation or cytotoxicity assays. Cell viability and anti-CD16- or ionomycin-induced Ca^{2+} fluxes were verified at the end of the incubation period of the killing assays (~4 hr after BAPTA-AM treatment).

LFA-1 Affinity

The assay was performed as detailed (Hoffmann et al., 2011). In brief, IL-2-activated NK cells and GFP-positive RMA-S cells were washed in ice-cold binding buffer (RPMI containing 0.5% bovine serum albumin, 1 mM $CaCl_2$, and 2 mM $MgCl_2$). They were then resuspended at 2×10^5 NK cells per 100 μl or 4×10^5 RMA-S cells per 100 μl. 100 μl of each cell type was then mixed and centrifuged at 1,500 rpm at 4°C. After removing 150 μl of supernatant, the remaining volume was supplemented with 5 μl (~2.5 μg) of Alexa 647-coupled ICAM-1-Fc (R&D Systems) or human IgG_1. After vortexing, cells were incubated at 37°C for 15 min. To stop the stimulation, ice-cold binding buffer was added and cells were centrifuged at 1,500 rpm at 4°C. Cells were finally resuspended in ice-cold binding buffer and analyzed by flow cytometry. As control, NK cells were stimulated with phorbol myristate acetate (PMA; 2 μM final concentration).

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures and six figures and can be found with this article online at doi:10.1016/j.immuni.2012.03.023.

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